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Research Article

TOPICAL APPLICATION OF *EUGENIA CARYOPHYLLUS* OIL AGAINST RINGWORM INFECTION OF HUMAN BEINGS

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ABSTRACT

Objective: At least one million people are suffering from dermatophytosis in India. These mycoses although normally not lethal are unpleasant, frequent disease recurrence, and resistant dermatophytic strains cause considerable economic losses. During the antifungal evaluation of essential oil of *Eugenia caryophyllus,* all concentrations were found to be an excellent inhibitor against selected fungi as compared to standard antifungal drugs griseofulvin, ketoconazole, and itraconazole. The present work deals with the preparation of an ointment from *E. caryophyllus* oil for the treatment of ringworm infection in human beings.

Materials and Methods: Due to these potent fungicidal properties, an ointment of *E. caryophyllus* was prepared and topically applied on tinea patients attending the outpatient Department of Skin, Venereology, and Leprology, SMS Hospital, Jaipur. Patients were diagnosed as tinea corporis, tinea capitis, tinea manuum, and tinea barbae. The medication was done twice a day for 3 weeks as advised by the skin specialist.

Results: All patients showed positive potassium hydroxide (KOH) results at the beginning of the trial. After the 2nd week of treatment, every patient was KOH negative and remained negative when re-examined after one month of treatment. All patients were completely cured within 3 weeks of the treatment.

Conclusions: Ointment showed excellent results, found cheaper substitutes to cure the disease without any adverse side effect. The present study offers a high possibility of complete cure of tinea infection and suggesting its uses as raw material by pharmaceutical industries for the development of antidermatophytic drug in prevailing conditions where dermatophytes are becoming resistant against popular antifungals.

Keywords: Dermatophytosis, Tinea corporis, Tinea barbae, Tinea capitis, Tinea manuum, Essential oil.

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INTRODUCTION

Dermatophytosis poses a serious concern to the sociologically backward and economically poor population of India. In dermatophytic infection of the skin, the fungus remains confined to the stratum corneum while pathogenic changes are produced in the deeper layer of the epidermis and dermis as per medical observation. These fungi produced a ring-shaped lesion of infected skin. Clinical surveys [1-4] carried out in India have shown ringworm as one of the most common dermatomycoses caused by the species of *Epidermophyton, Microsporum,* and *Trichophyton.* The disease is predominant in tropical and subtropical countries due to their, prevailing moisture and temperature regimes and pose a therapeutic problem [5,6]. Despite the availability of new systemic antifungal therapies, dermatophytic infections are difficult to eradicate completely, with recurrence reported in up to 25%–40% of cases [7]. Many antifungal synthetic drugs, namely terbinafine imidazoles and butenafine are found effective against dermatophytosis [8]. However, disease recurrence, resistant dermatophytic strains, and adverse effects are some drawbacks associated with popular antifungals.

In recent years, there has been a gradual revival of interest in the use of medicinal plants in developed as well as in developing countries, because herbal medicines have been reported to be safe and without any adverse side effect thus, a search for new drugs with better and cheaper substitutes from plant resources are a natural choice [9-14].

These finding promoted us to explore other plant products which could be exploited as effective antifungals. In our previous work, we explored *Trachyspermum ammi* oil against ringworm infection of human beings and all patients were cured successively without any adverse effect [15].

Recently, we have carried out the detailed antifungal study of the essential oil obtained from the flowering bud of *E. caryophyllus in vitro* as well as in clinical trials against ringworm infection in human beings. Eugenia or clove oil has been used as dental care product from the ancient time, but no work has been reported as dermal care product till now especially for ringworm treatment.

MATERIALS AND METHODS

Oil extraction

Clove flowering buds purchased from the local market of Jaipur city in April 2012 were identified by Prof. P. C. Jain, Department of Botany, University of Rajasthan, Jaipur. The semi-crushed buds were hydrodistilled in a Clevenger's apparatus for 4 h. Moisture free oil was then stored in amber colored bottles and kept in the refrigerator. The oil yield was 4.1% (v/w).

Microorganism for *in vitro* studies

Clove oil was evaluated for their antifungal properties against selected pathogens. *Trichophyton rubrum* and *Trichophyton simii* were isolated from infected skin scrapings of tinea patients from SMS Hospital, Jaipur, while *Chrysosporium tropicum* and *Chrysanthemum indicum* were isolated from soil samples through To. Ka. Va. hair baiting technique of Vanbreuseghem [16]. These fungi were maintained on Sabouraud's dextrose agar medium.

Screening of oil

The filter paper disc diffusion assay by Wannisorn *et al.* [17] was used with slightly modification for screening the essential oils against

dermatophytes. Standard size Whatman no. 1 filter paper discs 6.0 mm in diameter, sterilized by dry heat at 140°C in an oven for 1 h was used to determine antifungal activity. 20 ml sterilized Sabouraud's dextrose agar medium was taken in each autoclaved Petri dish and allowed to solidify. The fungal spore suspension was prepared in sterilized 0.85% saline water by transferring a loopful of 15 day-old culture. 1 ml of spore suspension of approximately 0.5–5 × 10^4 (cfu/ml) was spread over the respective agar medium plates. Sterilized filter paper was soaked in neat undiluted oil. An oil saturated disc was placed on an agar plate containing fungal spore suspension. Ketoconazole was used as a standard drug. These plates were incubated. Five replicates were kept in each case, and the average values were determined, and inhibition zone (IZ) was observed. The antifungal activity was determined by measuring the IZ around the disc. The activity of the oil was measured by the following formula.

$$\text{Activity Index } (\text{AI}) = \frac{\text{Inhibition Zone } (\text{IZ}) \text{ of samples}}{\text{Inhibition Zone } (\text{IZ}) \text{ of standard}}$$

Minimum inhibitory concentration (MIC) through semisolid agar antifungal susceptibility method:

Semisolid agar antifungal susceptibility testing method of Provine and Hadley [18] for endpoint determination (MIC) was carried out in BHIA (HiMedia). BHIA was prepared according to manufacturer's instruction.

Inoculum preparation

Sterile swab dipped into sterile tween 80 was used to pick the pure colony of yeast. This was then suspended in 3–4 ml of sterile normal saline and vortexed. The turbidity of the homogenous suspension was adjusted to ~0.5 McFarland standard. Similarly, inoculum was prepared for filamentous fungi (3–7 days old slant at 37°C on potato dextrose agar). By swabbing the pure colony (mixture of conidia and hyphal fragments) was suspended in 3–7 ml of sterile saline. The mixture was vortexed and heavy particles were allowed to settle. The homogenous suspension was adjusted to 0.5 McFarland standard.

Inoculation of drug-containing tubes

The semisolid agar tubes containing known concentrations of test oils as well as oil-free controls, prepared in duplicate, were inoculated with one loopful (HiMedia Flexiloop 4) of 0.5 McFarland adjusted culture by inserting the loop deep within the semisolid agar. The tubes were incubated at 37°C for 48 h (96 h for dermatophytes). A loopful of the inoculum suspension was streaked onto Sabouraud dextrose agar to check for purity and viability.

Endpoint determination

Endpoint determination was done according to the NCCLS/CLSI guidelines, M27-A, and M38-A. Growth was compared to that of oil-free control and scored by visual inspection as follows: +4: growth same as control; +3: slight decrease in growth; +2: significant reduction in growth reduction 80% in yeast and 50% in filamentous; +1 slight growth or few visible hyphal fragments; and 0: no growth.

Statistical analysis

Statistical analysis was carried by one-way ANOVA following multiple comparison tests Turkeys method. Data are expressed as the mean and standard error of the mean.

Ointment preparation and application

About 1% concentration of *E. caryophyllus* was (clove oil) prepared in petroleum jelly and labeled it as ointment EC.

A comparative efficacy of *E. caryophyllus* with different antimycotic drugs such as griseofulvin, itraconazole, and ketoconazole showed that all concentrations of *E. caryophyllus* were found to more effective than standard drugs. In our previous work Jain and Sharma, 2003, we also used 1% and 2% concentration of *T. ammi* oil against dermatophytes causing ringworm infection of human being and cured all patient successively. Before applying this ointment of tinea patients patch test of Roxburgh and Borrie [19] was followed to find out whether these ointments have any irritant activity or not.

Clinical application

For *in vivo* investigation, patients (male and female) of different age groups (infant to 50 years) were selected. All patients were voluntary. A total of 35 patients were selected for study to see the clinical response of ointment. This was applied topically on patients to control the fungal infection. The medication was done twice a day for 3 weeks as advised by the skin specialist. The patients were not allowed to take any other systemic or topical therapy during the course of the present study.

Patients were diagnosed for different clinical types such as tinea corporis, tinea capitis, tinea manuum, and tinea barbae based on site and type of infection. The diagnosis was further confirmed by microscopic examination of scrapings (from infected area) treated with 10% KOH. Only KOH positive cases were enrolled for the study. Patients were examined just before the start of therapy and at the end of each week of the treatment, up to their complete cure. At each visit of the patient, the same reference lesion was scraped for fungal culture to identify the organism and for demonstration of the presence of hyphae by microscopic examination of the scraping which was covered with 10% KOH preparation. Signs and symptoms of inflammation as erythema, scaling, itching, maceration, vesiculation, and pustulation were recorded as mild, moderate, severe, or absent. The fungal culture was identified by standard protocol.

Additional information of patients regarding their profession, living conditions, along with clinical diagnosis-site of infection, primary or secondary infection, sex, age were also recorded. Usually, patients with secondary infection were avoided for the study. Although the cutaneous fungal disease manifested itself in several body areas and all the affected areas were treated, only one was selected and designated

Table 1: Comparison of efficacy of *Eugenia caryophyllus* oil with commercial antifungal drugs

Concentrations of oil (%)	Test fungi													
	Trichophyton rubrum				*Trichophyton simii*				*Chrysosporium indicum*			*Chrysosporium tropicum*		
	IZ	AI			IZ	AI			IZ	AI		*IZ*	AI	
		TC/G	TC/I	TC/K		TC/G	TC/I	TC/K		TC/K			TC/G	TC/I	TC/K
25	25	0.893	1.190	0.490	18	0.750	0.9	0.486	30	1.765		28	0.8	1.647	0.718
50	47	1.679	2.238	0.921	36.33	1.514	1.816	0.982	42	2.470		36	1.028	2.118	0.923
75	75	2.679	3.571	1.470	55	2.292	2.75	1.486	50	2.941		52	1.495	3.078	1.342
100	95	3.393	4.524	1.863	71	2.958	3.55	1.919	57	3.353		58	1.648	3.392	1.479

IZ: Inhibition zone including 6 mm diameter of filter paper disc, AI: Activity index, TC: Test compound. Inhibition zones of standard griseofulvin (G) against *Trichophyton rubrum*=28 mm; *Trichophyton simii*=24 mm; *Chrysosporium tropicum*=35 mm. Inhibition zones of standard itraconazole (I) against *Trichophyton rubrum*=21 mm; *Trichophyton simii*=20 mm; *Chrysosporium tropicum*=17 mm. Inhibition zones of standard ketoconazole (K) against *Trichophyton rubrum*=51 mm; *Trichophyton simii*=37 mm; *Chrysosporium tropicum*=39 mm; *Chrysosporium indicum*=17 mm

as the reference lesion. At each visit of the patients, overall clinical improvement was reported as none, partial, significant, or completely clear by comparing the state of their infection with the state at the time of their initial visit. Adverse systemic or local reactions were noted (at each visit) and recorded as mild, moderate, or severe.

RESULTS

E. caryophyllus is very well studied nowadays. Lots of works have been done on the chemical composition of eugenia oil [20-24]. Eugenol is a major constituent of eugenia oil ranging from 82% to 90% according to collection time and cultivation place. Other components are trans-β-caryophyllene, eugenyl acetate, α-humulene, chavicol, β-caryophyllene oxide, α-cubebene, α-copaene, and δ-cadinene.

In vitro antidermatophytic activity

Four concentrations of Eugenia oil were prepared and applied against selected dermatophytes. Data were compared with the most commonly used standard allopathic drugs such as ketoconazole, itraconazole, and griseofulvin (Table 1). In the case of C. *indicum,* all four concentrations of clove oil exhibited more prominent inhibitory effects than ketoconazole. Griseofulvin and itraconazole were devoid of any antifungal effect against this fungus. In the case of *T. rubrum,* 100%, 75%, and 50% clove oil produced 95 mm, 75 mm, and 47 mm IZ, respectively, and had prominent efficacy in comparison to griseofulvin (IZ = 28 mm) and itraconazole (IZ = 21 mm). A comparative efficacy of this oil with ketoconazole against *T. rubrum* showed that 100% and 75% oil had excellent inhibitory properties (Activity index [AI] = 1.863 and 1.470, respectively) while 50% concentration showed slightly less effect (AI = 0.921). The same results were also seen in *T. simii* and *C. tropicum.* 100%, 75%, and 50% oil showed a more prominent effect than griseofulvin and itraconazole. 25% concentration also showed stronger effect than itraconazole in the case of *C. tropicum* (AI = 1.647) and was slightly comparable with itraconazole in the case of *T. simii* (AI = 0.9). Maximum zone of inhibition 95 mm was recorded against *T. rubrum* in the presence of pure oil (Fig. 1).

MIC of clove oil

In present investigation, MIC of clove oil against seven selected fungi, namely *Candida albicans, T. rubrum, T. verrucosum, M. gypseum, M. canis, M. fulvum,* and *F. verticilloides* was carried by SAAS method (Table 2). Clove oil showed excellent antidermatophytic activity against all test fungi. MIC of clove oil against *T. rubrum* was found to be 0.05 ± 0.000 µl/ml followed by 0.1 µ/ml for *C. albicans, M. gypseum, M. canis, T. verrucosum,* and *M. fulvum*. MIC for *F. verticillioides* was found to be 0.2 ± 0.002 µl/ml.

In vivo experiment

Data incorporated in Table 3 show that all the 35 patients of tinea infection recovered completely with the timely application of these ointments (Figs. 2 and 3).

Tinea corporis

A complete treatment period of tinea corporis differs (4–6 weeks) with the different allopathic drug. Out of 15 patients treated with ointment EC, three showed complete relief within 1 week, nine patients were completely cured in the 2nd week, and the remaining showed complete cure during the 3rd week of treatment. *Trichophyton rubrum* and *T. simii* were the main causative agents of tinea corporis.

Table 2: Minimal inhibitory concentration (µl/ml) of essential oils against selected dermatophytes

Fungal species	MIC
Candida albicans	0.1±0.000
Microsporum gypseum	0.1±0.033
Microsporum canis	0.1±0.002
Trichophyton rubrum	0.05±0.000
Trichophyton verrucosum	0.1±0.000
Microsporum fulvum	0.1±0.033
Fusarium verticillioides	0.2±0.002

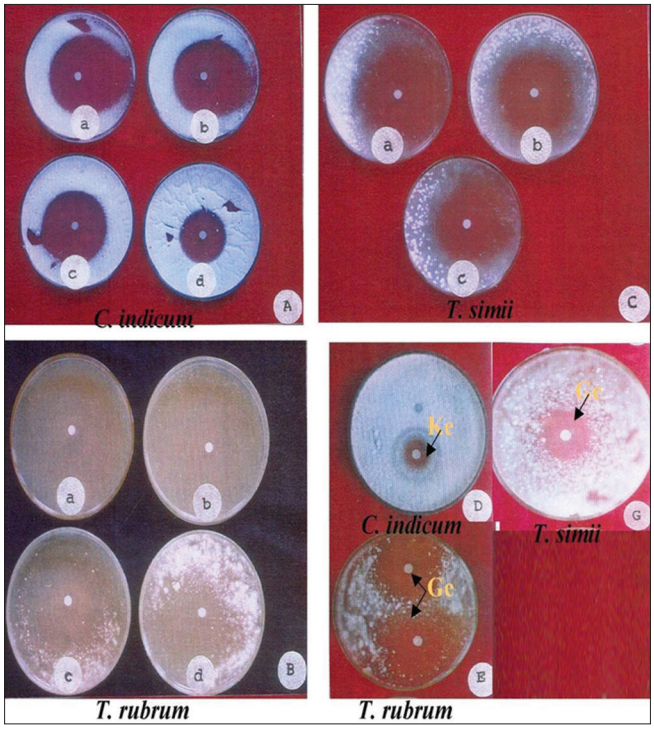

Fig. 1: Efficacy of *Eugenia caryophyllus*

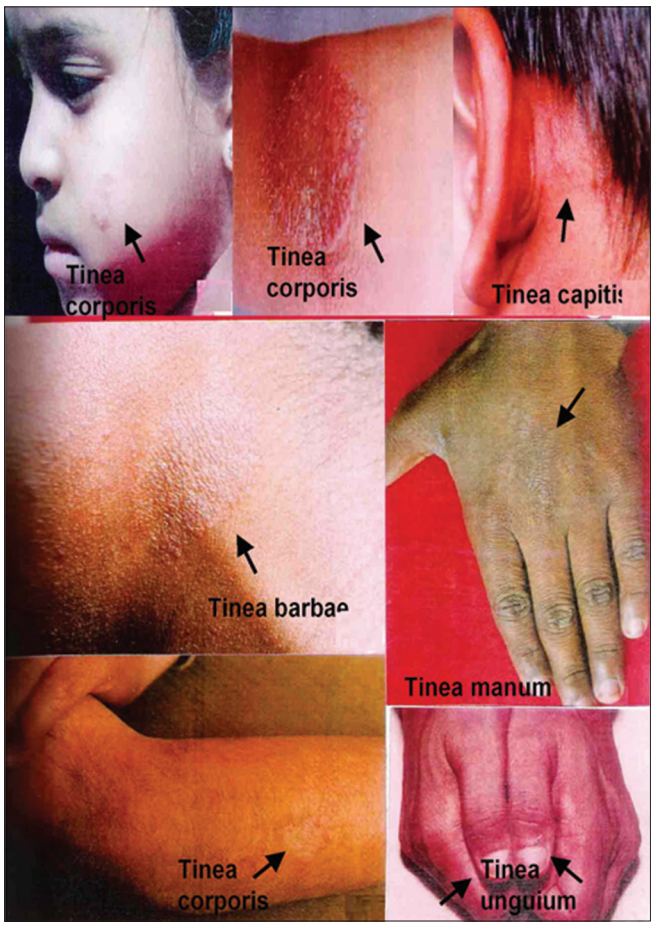

Fig. 2: Showing different clinical types of tinea infection

Table 3: Response of ointment EC on tinea infections

Number	Clinical diagnosis	Fungal species isolated	Number of patients	Worse	No improvement	Number of patients showing improvement								
						Partial			Significant			Crude completely		
						1st week	IInd week	IIIrd week	1st week	IInd week	IIIrd week	1st week	IInd week	IIIrd week
1.	Tinea corporis	*Trichophyton simii*	6	-	-	-	-	-	3	3	-	-	3	3
		Trichophyton rubrum	9	-	-	-	-	-	6	-	-	3	6	-
2.	Tinea capitis	*Trichophyton rubrum*	6	-	-	6	-	-	-	6	-	-	-	6
3.	Tinea manuum	*Trichophyton rubrum*	9	-	-	6	-	-	3	9	-	-	-	9
4.	Tinea barbae	*Trichophyton rubrum*	5	-	-	-	-	-	5	-	-	-	5	-

EC: Eugenia caryophyllus

Fig. 3: Treatment of different clinical types with ointment EC

Tinea capitis

A total of six patients of tinea capitis were treated with ointment EC. All patients showed significant improvement in the 2nd week of treatment and cured completely within 3 weeks without any adverse side effect.

Tinea manuum

A complete treatment period of tinea manuum is 6–8 weeks with different drugs. In present investigation out of nine patients of tinea manuum, three patients showed significant improvement in 1st week, and rest six in 2nd week. All patients completely cured within 3 weeks.

Tinea barbae

Five patients of tinea barbae suffering from *Trichophyton rubrum* showed significant improvement in 1st week of treatment and completely cured in the 2nd week of treatment.

During the present study, all patients of ringworm infection were completely cured in 12–18 days of treatment without any adverse side effect. Ointment, when tested for its irritation activity on experimental animals, did not show any irritation effect. The same result also observed during the patch test method on a human being.

DISCUSSION

During present investigation, *E. caryophyllus* oil exhibited excellent antidermatophytic activity as compared to standard allopathic drugs

such as griseofulvin, ketoconazole, and itraconazole. Antifungal and antidermatophytic properties of clove were also reported [25,26]. Therefore, 1% concentration of oil was used to prepared ointment EC.

A complete treatment time of the different clinical type of tinea infection differs from 4 to 10 weeks with different allopathic drugs. During treatment, all patients of ringworm infection were completely cured in 12–18 days of treatment without any adverse side effects. Out of 15 patients of tinea corporis, three cured completely within a week, nine after the 2nd week of treatment and the remaining were cured after 3rd week of treatment. Six patients of tinea capitis and nine of Tinea manuum were completely cured in 3rd week of treatment. Likewise, all patients of tinea barbae were cured in the 2nd week of treatment. Ointment, when tested for its irritation activity on experimental animal, did not show any irritation effect. The same result also observed during the patch test method on human being.

In our previous work [15], we explored *T. ammi* essential oil against dermatophytes and found excellent results. During the comparative analysis of both oils, Eugenia oil exhibited more prominent antidermatophytic activity (laboratorically and clinically) as compared to *T. ammi* oil. Shahi *et al*. [27] also used 1% concentration of *Eucalyptus citriodora* oil against dermatophytic infection of a human being. At the end of treatment, 55.5% of patients recovered completely and 45.5% of them showed significant improvement. Shahi *et al.* [28] used 1.0 µl/ml concentration of *Eucalyptus pauciflora* against selected human pathogenic fungi and prepared an ointment (1% v/v) for ringworm infections of human beings. At the end, 60% of patients recovered completely and 40% showed significant improvement from the disease.

CONCLUSION

The oil of *E. caryophyllus* due to its strong antifungal activity inhibiting heavy doses of inoculum having fungicidal properties and with no irritation on human skin can be used successfully in the form of broad-spectrum antimycotic drug for the control of superficial fungal infection in human beings. *E. caryophyllus* also exhibited stronger antidermatophytic compound as compared to all synthetic antifungal drug prescribed by doctors during the course of treatment. These have been found to be better and effective substitutes for curing the disease without any adverse side effect. They are easy to apply and respond much faster than synthetic allopathic preparations. Isolation and identification of active principles of *E. caryophyllus* at different temperature interval for antidermatophytic activity are in progress.

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AUTHORS' CONTRIBUTIONS

Dr. Neetu Jain carried out all the experiments while Dr. Meenakshi Sharma designed the experiments and help in the preparation of the manuscript.

CONFLICTS OF INTEREST

No conflicts of interest.

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